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The Role of Zinc in the Insulin Metabolism in the Pancreas of the Rat

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THE ROLE OF ZINC IN INSULIN METABOLISM IN THE PANCREAS OF THE RAT

by

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To my parents Mr. and Mrs. C. Y. Liaw

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ABSTRACT

The Role of Zinc in Insulin Metabolism

in the Pancreas of the Rat

by

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A series of in vitro trials have been conducted to determine what relationship exists between zinc and the insulin mechanism.

There were no significant differences of the zinc contents in total pancreases excised from the rats thirty minutes after the intraperitoneal glucose dose or from the non-dosed controls. When pancreases were cut into three pieces, and treated in three ways: Incubated without glucose (IWOG) followed by isolation of the islets; incubated with glucose (IWG) followed by isolation of the islets; islets were isolated, then incubated with glucose (IWG). The zinc content in the islets was significantly higher ($p<0.01$) in the first group than the others.

The insulin release and zinc movement were studied in zinc-deficient status. In both Experiment 3 and 5 each pancreas was divided into two, one was treated IWG, one was treated IWOG. There were significant ($p<0.01$) differences of zinc content in the islets between IWG and IWOG groups in zinc-supplemented pair-fed and ad lib. controls but not in zinc-deficient rats. In Experiment 5 the zinc contents in the islets of IWOG group were significantly ($p<0.01$) lower in zinc-deficient rats than in

zinc-supplemented pair-fed rats. Insulin release from the islets incubated with glucose was significantly ($p<0.01$) less in zinc-deficient group than in zinc-supplemented pair-fed group. Glutathione contents of the pancreases were measured in Experiment 3, no significant difference was found between zinc-deficient group and both zinc-supplemented groups.

Rats were intraperitoneally dosed with glucose or with saline in Experiment 6. After 30 minutes pancreases were excised and the isolated islets were treated with IWG or IWOG. There was no significant difference of zinc content in the islets treated IWOG in glucose-dosed and saline-dosed groups, but significant ($p<0.01$) difference was found in the islets treated IWG. Insulin release from the islets treated IWG was significantly ($p<0.01$) less in glucose-dosed group than in saline-dosed group.

It is concluded that zinc is released from the islets as a component of insulin on glucose stimulation, zinc-deficiency results in an impairment of insulin release or synthesis, and insulin secretion was reduced in the isolated islets taken from animals prestimulated with glucose in vivo.

(65 pages)

INTRODUCTION

An impairment of glucose tolerance was observed in zinc-deficient rats after the administration of either intravenous or intraperitoneal glucose dose. (Quarterman et al., 1966; Hendricks et al., 1972). No impairment was found after oral glucose dosing by Hove (1937) and Henkricks et al. (1972). The latter observations suggested that the orally administered glucose was more efficient in releasing insulin from the pancreas. Fasel et al. (1970) have postulated a factor, produced by the duodenal mucosa after oral glucose stimulation, that aids in the release of insulin from the pancreas.

Insulin has a strong affinity for zinc, and exists in the form either devoid of zinc or in combination with zinc, but both are physiologically active. It is believed that zinc is linked to the metabolism of insulin in the beta cells of the pancreas (Scott 1934). In zinc-deficient animals, the zinc content in the pancreas was reduced (Sullivan et al., 1974), concomitant with a reduced granulation of beta cells of the pancreas was observed (Boquist et al., 1969). A decreased zinc content in the beta cells of the pancreas has been reported after glucose administration (Toroptsev et al., 1972). Gershoff et al. (1973) found that pancreatic insulin was not reduced, but circulating insulin was reduced in zinc-deficient rats. Also there was a decreased release of insulin from the zinc-deficient pancreas on in vitro glucose stimulation. He suggested zinc deficiency resulted in an impaired insulin release from the pancreas. Controversially, serum insulin levels in zinc-deficient hamsters reported are unaffected (Boquist et al., 1969).

Hsu et al. (1968) reported that the contents of non-protein SH compounds and glutathione in the livers of zinc-deficient rats were higher than those of zinc-supplemented controls. This may implicate the possibility of increased insulin degradation on zinc deficiency.

In this research a series of in vitro trials were undertaken to investigate the role of zinc in insulin secretion. Also glutathione contents in the pancreases were measured to investigate whether there is a change of glutathione content in the pancreas of zinc-deficient rats, which may be responsible for the insulin degradation.

REVIEW OF LITERATURE

Glucose Stimulation of Insulin Secretion from the Pancreas

Insulin is synthesized and stored in the beta cells of the islets of Langerhans of the pancreas. It's secretion is influenced by various factors, such as ACTH, epinephrine, glucose, glucagon, adrenocortical hormones, etc. Of the above glucose is a major physiological stimulus, which elicits insulin secretion in vivo, in in vitro preparations of perfused pancreas and of pancreas pieces, and in in vitro preparations of isolated islets of Langerhans.

It has been questioned recently whether the insulin secreted in response to glucose is derived exclusively from performed secretory granules, or derived also from the site of biosynthesis. Jamieson et al. (1967 a, b), Howell et al. (1969) and Baiton et al. (1970) in their radioautographic and histochemical studies on the biosynthesis and intracellular transport of secretory insulin in response to glucose stimulation, strongly supported that the insulin secretion is localized within the rough endoplasmic reticulum, Golgi apparatus and the secretion granules. Since the endoplasmic reticulum is site for producing insulin, so that it seems the insulin secreted is derived also from the site of biosynthesis. There is little evidence that glucose has a direct and primary effect on insulinogenesis. Seltzer et al. (1962) found more insulin secreted from dog pancreases in situ after a glucose dose than after a tolbutamide dose, and concluded that glucose was stimulating insulin synthesis, but there was no chemical evidence provided.

Stephen et al. (1972) investigated the in vitro effects of glucose and analogs of glucose on insulin secretion in the rat and mouse islets. They showed that the curves relating rates of insulin secretion to glucose concentration were sigmoid and tended to V_{max} at approximately 20 mM glucose. Mannose and glucosamine were the only analogs of glucose which stimulated secretion in the absence of glucose. Grodsky et al. (1963), in experiments with isolated perfused pancreas of rats, showed that the stimulatory effect of glucose on insulin release was at a secretion rate proportional to the amount of glucose perfused. A similar quantitative dependancy of insulin secretion on glucose concentration was observed in rabbit pancreas segments. (Coore et al., 1964).

Stephen et al. (1972) assumed that glucose might interact directly with the beta cells of the islets to excite secretion. Two mechanisms were suggested to be considered, either direct interaction of glucose with a receptor molecule which is thereby enabled to excite the secretion process (regulator site mechanism) or interaction of a metobolite of glucose with a component of the secretion mechanism leading to excitation (substrate site mechanism). Valdemar et al. (1974) reported, in isolated rat pancreatic islet incubates, D-glucose stimulated the accumulation of cAMP, but had no effect on cAMP accumulation in the rat adipocytes, and concluded that the action of glucose was specific in the islets. He also found that over a range of glucose concentrations (0.6 to 7.0 mg per ml), the amount of released insulin after a 60 minute incubation period was well correlated with medium cAMP content, and cAMP accumulation seemed to precede the initiation of insulin release, so that he concluded that the insulin release induced by glucose may be mediated by cAMP

in the beta cells. Coore et al. (1963) observed that glucose stimulation of insulin secretion was blocked by inhibitors of its metabolism, such as mannoheptulose. In another study made in 1964, he showed glucose stimulation of insulin secretion was inhibited by p-phenylenediamine and phenazine methosulfate, although these agents stimulate the oxidation of the hexose monophosphate pathway. Grodsky et al. (1963) found that the action of glucose stimulation was not inhibited by the complete blocking of insulinogenesis with dinitrophenol, and concluded that glucose acts directly on the mechanism controlling the release of stored insulin.

The Role of Zinc in the Metabolism of Insulin

Insulin has a strong affinity for zinc and is associated with this metal in the islets of Langerhans of the pancreas. Vallee et al. (1959) in his study on biochemistry, physiology and pathology of zinc, suggested that one zinc atom probably chelates two imidazole residues of amorphous insulin, while in crystalline insulin, carboxylate groups are postulated to bind additionally, and reported that insulin could be prepared in both amorphous and crystalline forms entirely free of zinc, but still physiologically active. Scott et al. (1934) reported, in some concentrations of zinc, the formation of crystalline insulin was greatly facilitated.

Sheline et al. (1943) found that the zinc content in the pancreas was higher than other tissues. McIsaac et al. (1955), with a method of autoradiography to study the retention and distribution of radioactive zinc-65 in the rat pancreas, found zinc-65 entered both islets and acinar tissues, but the acinar cells lost the radioisotope quickly, while

the concentration in islet cells remained high and constant throughout the 92 hours of observation. They suggested zinc mostly locates in the islet cells. Logothetopoulos et al. (1964) reported that zinc was mostly localized in the beta cells of the islets of Langerhans by comparing the concentrations of characteristic dark-staining granules in different cells. Shevchuk et al. (1964) found, following diabetogenic doses of alloxan, the beta cells lost their zinc entirely, while the alpha cells increased in both number and zinc content. Williams et al. (1970) and Sullivan et al. (1974) found that there was a reduction of the zinc content in the pancreas when the rats were fed with zinc-deficient diets. Boquist et al. (1969) also reported that there was a reduced granulation of beta cells of the pancreas in zinc-deficient hamsters. Toroptsev et al. (1974) reported that starvation was accompanied by considerable accumulation of zinc in the beta cells of the pancreas in rats, and the zinc content in beta cells was sharply reduced after administration of glucose, while the zinc content in the alpha cells increased a little.

Quarterman et al. (1967) found the zinc ion in the incubation media at concentrations similar to that found in plasma and tissues stimulated the uptake of glucose by adipose tissue. In a later paper published in 1969, he reported that at low concentrations of both zinc and insulin in the incubation media, the increase of glucose uptake by the adipose tissue was additive and suggested that a synergistic effect may be apparent at low concentrations of zinc and insulin. But Huber et al. (1966) found that zinc, at concentrations in the range of those observed

in human plasma, inhibited the activity of isolated rat epididymal adipose tissue in the presence of insulin and glucose in the incubation media.

Quarterman et al. (1966) found that after a single intraperitoneal glucose dose, blood glucose levels peaked higher and later in the zinc-deficient rats than in the zinc-supplemented rats. When a second intraperitoneal dose of glucose was given one hour after the first, the zinc-deficient rats had a marked elevation in blood glucose in comparison to the control rats. He suggested that the rate of secretion of insulin by the zinc-deficient rats in response to a glucose stimulus may be less than that of the control ones. Hove et al. (1937) found only small differences in oral glucose tolerance between zinc-deficient and the control rats. Hendricks et al. (1972) showed that zinc-deficient rats had a singificantly ($p < 0.01$) abnormal glucose tolerance to an intraperitoneal glucose dose, but not to an oral dose. They suggested there was probably an increased rate of insulin degradation in the zinc-deficient rats after an intraperitoneal glucose dose, or the mechanism by which intraperitoneal glucose stimulates the release of insulin was impaired in the zinc-deficient rats. As to the fact that there was no impairment of glucose tolerance in zinc-deficient rats after an oral administration, they explained the increased insulin secreted in response to the intestinal stimulus of the glucose dose would take longer to degrade in the liver than the insulin released after the intraperitoneal dose. On the contrary, Macapinlac et al. (1966) were not able to show an effect of zinc deficiency on glucose tolerance in the rats after intraperitoneal glucose dose. Theurer et al. (1966) found there was no

difference in the oxidation of glucose between zinc-deficient and zinc-supplemented rats injected intraperitoneally with glucose dose. Quarterman et al. (1972) found the glucose tolerance of zinc-deficient rats after an intraperitoneal glucose dose was not different from that of rats given the zinc-supplemented diet. In his experiment, the tolerance test of each rat on the zinc-supplemented diet was measured a day after its zinc-deficient pair-mate so that the two animals were comparable with regards to both feeding-pattern and the preceding day's food intake. He suggested the impaired glucose tolerance in zinc-deficient rats found by other workers was attributed to a difference in feeding pattern between zinc-deficient rats eating slowly and continuously and their meal-fed pair-mates which ate their food over a relatively short period.

Quarterman et al. (1966) showed that zinc-deficient rats had less plasma insulin than their pair-fed controls (mean values 24 ± 5 μu insulin equivalent/ml and 30 ± 4 μu/ml) and found zinc-deficient rats had a much lower content of saponifiable lipid in their tissues and a much higher fasting free fatty acid level in plasma (increased from 423 μequiva./1 in rats given zinc to 1050 μequiva./1 in zinc-deficient rats). He also found zinc-deficient rats were less sensitive to insulin. The zinc-supplemented rats became comatose or convulsed within an hour of intraperitoneal injection of insulin, while zinc-deficient rats entered a coma three hours after injection. Swenerton et al. (1968) found that the plasma zinc concentration fell extremely rapidly when the rat was fed on zinc-deficient diet. Quarterman et al. (1972) showed the plasma insulin concentration in zinc-deficient rats after an intraperitoneal dose of glucose tended to be lower than in those given a zinc-supplemented diet. Gershoff et al. (1973) found that feeding of high or low zinc

diets did not alter the insulin content of the pancreas, but immunoreactive serum insulin and the total serum insulin-like activity measured by an in vitro adipose itssue assay were significantly ($p < 0.01$) reduced in the zinc-deficient group compared with the ad libitum controls, and the pair-fed control group also showed significantly ($p < 0.05$) reduced circulating immunoreactive insulin but not the total serum insulin-like activity. He also found that, in vitro studies of the release of pancreatic insulin during short incubation periods with glucose, significantly ($p < 0.001$) less immunoreactive insulin as well as insulin-like activity was released by pancreata from zinc-deficient rats compared with pair-fed and ad libitum control rats. The pair-fed group showed a significant ($p < 0.01$) decreased release of pancreatic insulin compared with ad libitum-fed controls. On the contrary, Boquist et al. (1969) indicated no difference of serum insulin content was found between zinc-deficient and zinc-supplemented hamsters, whether fasted or following an intravenous dose of glucose.

Insulin Degradation

Approximately 50 percent of the insulin secreted into the pancreactic vein is removed and degraded during a single passage through the liver (Mortimore et al., 1959; Madison et al., 1959). Once in the circulation, insulin is rapidly cleared at a rate of two percent per minute (Berson et al., 1956). The degradation of insulin is important in insulin Metabolism.

Vigneaud et al. (1931) found that crystalline insulin was completely inactivated by cysteine and glutathione in the liver homogenates. Mirsky et al. (1957) had a hypothesis that a single enzyme with a relatively high degree of specificity for insulin is present in the liver, upon incubation of insulin in phosphate buffer with a sulfhydryl compound, such as glutathione, the addition of this enzyme greatly increases the rate of insulin degradation. Narahara et al. (1959) reported that liver extracts could accelerate the reduction of insulin, and suggested that there are two enzymatic mechanisms which are responsible for this reduction.

(1) $\text{Insulin} + 2\text{GSH} \xrightarrow{\text{dialyzed liver enzyme}} \text{reduced insulin} + \text{GSSG}$

(2) $\text{GSSG} + \text{TPNH} + \text{H}^{+} \xrightarrow{\text{Glutathione reductase}} 2\text{GSH} + \text{TPN}^{+}$

Tomizawa et al. (1959) isolated a beef liver enzyme, which degraded insulin in the presence of sulfhydryl compounds of a low molecular weight, such as reduced glutathione, or 2-mercaptoethanol. In 1962, they reported the A chain of insulin was identified as the only trichloroacetic acid-soluble product from insulin in an incubation medium, in which insulin was degraded by GSH in the presence of the isolated liver enzyme, and concluded the enzyme promotes the reductive cleavage of disulfide bonds of insulin by small sulfhydryl compounds. In 1965, they isolated the enzyme from the human liver, and named it glutathione-insulin transhydrogenase. Varandani et al. (1966) isolated an enzyme from beef pancreas, which also could catalyze the reductive cleavage of the disulfide bonds of insulin. He suggested the requirement for glutathione is not specific, but a sulfhydryl-containing compounds must be present in order for the enzyme to catalyze the reduction reaction. In 1972, Varandani isolated glutathione-insulin transhydrogenase from the rat

liver. By using a tracer [^{125}I] insulin which was incubated with purified rat liver enzyme and GSH, he indicated that the trichloroacetic acid (TCA) soluble product formed from insulin was A chain. Chandler et al. (1972) reported that glutathione-insulin transhydrogenase is ubiquitous, it was found in 13 tissues tested in rats (pancreas, liver, intestine, spleen, kidney, testis, thymus, fat, lung, brain, heart, diaphram, skeletal muscle). They also found that the concentrations of the enzyme in the various tissues appeared to be inversely related to the insulin sensitivity of tissues, i.e. those tissues (muscle, diaphram, heart and fat) known to be highly sensitive to insulin stimulation of glucose uptake in vitro were found to contain the lowest concentrations of the enzyme's activity. It is suggested that glutathione-insulin transhydrogenase is the major insulin degrading activity present in the animal.

Katzen et al. (1966) reported under suitable conditions, glutathione-insulin transhydrogenase can facilitate the reverse reduction, i.e. the oxidation of protein sulfhydryl groups to reform disulfide bonds. Berson et al. (1957) showed that most of the resulting A and B chains from insulin are degraded by hydrolysis, since labeled iodotyrosine rapidly appeared after the injection of insulin-^{125}I. Langdon et al. (1960) found the A chain of insulin inhibits glutathione reductase. Randle et al. (1964) suggested, if operated in vivo, this inhibition will delay the rate of reformation of any GSH oxidized to GSSG, and so of further cleavage of insulin disulfide bonds by the thiol, consequently, fresh A chains will not be produced and enzyme activity will be released again. Langdon et al. (1961) found in the presence of small amounts of

$NADPH_2$, rat liver glutathione reductase was inhibited by small amounts of reduced B chain, and in turn the production of B chain was reduced. They suggested that it is a feed-back mechanism. Ensinck et al. (1964) showed that, after the incubation of insulin-^{125}I in serum with preparations of partially purified glutathione-insulin transhydrogenase, two radioactive compounds resulted, which had characteristics similar to the A and B chains of insulin, the B chain was combined with serum albumin, whereas the A chain was unassociated with any serum macromolecule. They concluded that the two chains are transported in mammallian extracellular fluids, and the serum albumin may act as a carrier protein for the B chain. Varandani et al. (1972) showed that the degradation of insulin is in a sequential order; first, a splitting of insulin into A and B chains by glutathione-insulin transhydrogenase, then followed by proteolysis of the resulting polypeptides to small molecular weight components. In 1973, he reported this sequential mechanism of the insulin degradation was also found in kidney, heart, and skeletal muscle homogenates of rat, suggested that this sequential degradation is probably a wide-spread mechanism. In the paper published the same year, he reported glutathione-insulin transhydrogenase is primarily located in the microsomal fraction of the rat liver homogenate, while the enzyme that further degrades insulin by proteolysis is located mainly in the soluble fraction, a significant amount of protease activity is also present in the mitochondrial fraction. He suggested that protease acts upon the intermediate products of insulin degradation, e.g. A and B chains of insulin, rather than upon the intact insulin molecule itself.

Glutathione, synthesized in the liver and accounting for almost all the erythrocyte non-protein thiol, plays an important role in insulin degradation by reducing the disulfide bonds of insulin. Glutathione exists dominantly in the reduced form, only two percent of the total glutathione is in oxidized form. The reduction of oxidized glutathione is mainly catalyzed by glutathione reductase. $NADPH_2$ is necessary for this reduction, which is formed from NADP by the reduction coupled to the enzymic oxidation of glucose-6-phosphate to 6-phosphogluconic acid (hexose monophophate pathway, HMP). Jacob et al. (1966) reported that increasing the ratio of oxidized glutathione to reduced form will increase the rate of HMP pathway metabolism.

Krahl et al. (1953) reported that liver slices, obtained from the diabetic rat, showed a diminished rate of incorporation of glycine-^{14}C into glutathione and protein, and the in vitro addition of both insulin and glucose were required in order to restore the rate of glutathione synthesis to normal. He concluded insulin plays a role in glutathione synthesis.

The liver glutathione content in the animal body may bear a significance in the regulation of insulin degrading activity, and it can be controlled by some factors. Edwards et al. (1952) showed that the glutathione content in the liver reduced to 40 percent of the normal amount after the rats were fed on a protein-free diet only one day. When protein was restored to the diet, the glutathione content not only rose, but even overshot the original value. Tateishi et al. (1974) showed on starving rats for one or two days, that the liver glutathione contents of the rats decreased to half of the normal level. On feeding

these animals again, the glutathione contents rapidly returned to normal. They concluded the maintenance of liver glutathione in rats depends upon their food intake. Smith et al. (1962) reported after a partial hepatectomy, regenerating liver showed a rise in its GSH, in contrast, the protein SH levels fell to nearly half the original value. Hsu et al. (1968) showed the concentrations of non-protein SH compounds and glutathione in the livers of zinc-deficient rats were significantly ($p < 0.01$) higher than those of zinc-supplemented controls. These changes were observed as early as one week of the experimental feeding, and no further increase of liver glutathione was noted when rats were deprived of zinc for two or three weeks. They also showed when incorporation of glycine-1-^{14}C into liver glutathione, the mean value of total glutathione in the livers of zinc-deficient rats was 60 percent more than that of zinc-adequate animals.

EXPERIMENTAL PROCEDURE

Experimental Design

Six experiments were conducted to study the relationship between zinc, glutathione and insulin secretion from the rat pancreas.

Experiment 1. Effect of intraperitoneal glucose dose on zinc content of the pancreas.

Experiment 2. Effect of in vitro glucose incubation on zinc movement from the islets of Langerhans of the pancreas.

Experiment 3. Effect of zinc deficiency on glutathione content in the pancreas and on zinc movement from the islets of Langerhans in response to glucose stimulation.

Experiment 4. Effect of in vitro glucose incubation on insulin release from the islets of Langerhans of the pancreas.

Experiment 5. Effect of zinc deficiency on insulin secretion mechanism.

Experiment 6. Effect of intraperitoneal glucose dose on the insulin secretion mechanism.

Animal Care

All rats were housed individually in stainless cages in a clean, ventilated and temperature-controlled room, and fed from glass feed cups and water bottles. Papers underneath the cages were changed every day.

The rats for zinc deficiency study were kept in a special small room designed for trace element study. The walls and floor were washed,

the racks, cages, feed cups and water bottles were carefully washed with EDTA solution. Papers with wax backing were used underneath the cages, and the decks were scrubbed each week. All rats were watered ad libitum with deionized water. The composition of basal low zinc diet is on Table 1. The zinc-supplemented diet was prepared by adding zinc

Table 1. Composition of basal diet

Ingredients	g/100g of Diet
Glucose	59.0
Dried egg white	15.0
Corn oil	10.0
EDTA-washed casein	5.0
α-cellulose	5.0
Mineral Mix[1]	4.0
Vitamin Mix[2]	2.0

[1]See Appendix A.
[2]Vitamin Diet Fortification Mixture, Nutritional Biochemical Corp., Cleveland, Ohio. See Appendix B.

sulfate ($ZnSO_4 \cdot 7H_2O$) to the low zinc diet to give final concentration of 100 ppm zinc. All diets were analyzed for zinc by the atomic absorption flame spectrophotometer. The basal low zinc diet used in Experiment 5 contained 1.75 ppm zinc.

The rats for zinc deficiency study were divided into three groups. One group was fed with zinc-supplemented diet ad libitum, the other two

groups were paired. They were divided into pairs as nearly equal in body weight as possible. One group was fed with zinc-deficient diet, the other group was fed with zinc-supplemented diet but each received the same amount of diet consumed the day before by the pair zinc-deficient rat. All rats were weighed weekly, and blood was drawn once a week for determination of serum zinc level.

Tissue Preparation

Isolation of the islets of Langerhans from the pancreas

According to Lacy's method (1967), immediately after the rat was decapitated, the common bile duct was cannulated with a polyethylene catheter near the hilus of the liver and the distal end of the common bile duct was clamped adjacent to the duodenum; 7.0 ml of Hank's solution was injected into the common bile duct to disrupt the acinar tissue of the pancreas.

The whole pancreas was removed, trimmed and washed in two changes of Hank's solution. The pancreas then was cut into small pieces with scissors and placed in a freshly prepared solution of collagenase (50 to 60 mg dissolved in 5 ml of Hank's solution). The tissue was stirred in a closed 15 ml flask using a magnetic stirrer and incubated 20 minutes at 37°C.

After incubation of the pancreas with collagenase, the mixture was diluted with 15 to 25 ml of Hank's solution in a graduate cylinder and allowed to settle for one minute. The intact islets would settle to the bottom of the cylinder and the supernatant was decanted and discarded.

The sediment containing the islets was resuspended in Hank's solution and centrifuged at 1500 RPM for five minutes. The supernatant was then decanted and the procedure was repeated at least eight times.

Experimental Sequence

Experiment 1. Effect of intraperitoneal glucose dose on zinc content in the pancreas

Twelve fasted male rats of the Sprague-Dawley strain were randomly divided into two groups. Six were intraperitoneally injected with 250 mg glucose per 100 gm body weight thirty minutes prior to autopsy. The other six were not dised. The pancreases were excised for total zinc analysis.

Experiment 2. Effect of in vitro glucose incubation on zinc movement from the islets of Langerhans of the pancreas

Five fasted male Sprague-Dawley-derived rats weighing 200 gm were sacrificed and pancreas were obtained.

Each pancreas was divided into three pieces for following treatments. (A) group was non-incubated, the islets of Langerhans were isolated. (B) group was cut into small pieces, incubated with glucose solution [Krebs-Ringer bicarbonate buffer containing 3 mg glucose per ml, gassing with CO_2/O_2 (5:95)] in a Dubnoff shaker at 37°C for 30 minutes, then followed the isolation of the islets of Langerhans. The islets of Langerhans of the (C) group pancreases were isolated first, then incubated with glucose solution as (B) group. The zinc content in the islets was analyzed.

Experiment 3. Effect of zinc deficiency on glutathione content in the pancreas and on zinc movement from the islets of Langerhans in response to glucose stimulation

Thirty-eight weanling male rats of the Sprague-Dawley strain were assigned to three groups. Ten were fed with zinc-supplemented diet ad libitum, the other 28 were divided into 14 pairs. One group was fed with zinc-deficient diet, the other group was pair-fed with zinc-supplemented diet.

Four weeks later, the serum zinc level of zinc-deficient group rats were less than 70 μg/100 ml, which indicated the animals were zinc-deficient enough.

All rats were decapitated after overnight fasting, and the pancreas was excised from each rat. Each pancreas was then divided into three parts, one for total and oxidized glutathione analysis (Tietze et al., 1969), and the remaining two parts for zinc analysis of the islets of Langerhans after non-glucose or glucose incubation.

Experiment 4. Effect of in vitro glucose incubation on insulin release from the islets of Langerhans

Two fasted male rats were sacrificed to get the pancreases. The islets were isolated by collagenase and pooled in 5 ml of Krebs-Ringer buffer, then evenly divided into two parts. One part was incubated in the glucose solution (4 mg/ml) at 37°C for 30 minutes, the other part was incubated in media without glucose. After centrifuging, the supernatant was obtained for the biological insulin assay. 2.5 ml of Krebs-ringer buffer containing the same glucose concentration (4 mg/ml) were made for the blank.

Experiment 5. Effect of zinc deficiency on the insulin secretion mechanism

Twenty-four male rats of the Sprague-Dawley strain weighing 80 gm were divided into two groups. One group was fed with zinc-deficient diet, the other group was pair-fed with zinc-supplemented diet. After three weeks, the serum zinc levels of zinc-deficiency rats were lower than 60 μg/100ml. It showed they were zinc-deficient enough.

The rats were decapitated after overnight fasting, and the pancreas was excised from each rat. The islets of Langerhans were isolated from each pancreas, and pooled in 2 ml Krebs-Ringer bicarbonate buffer, then evenly divided into two parts. One was incubated in glucose solution (glucose 4 mg/ml) at 37°C for 30 minutes, the other was incubated in media without glucose. By means of centrifuging, the islets were separated from the media and ashed for zinc analysis, and the media after glucose incubation were ready for biological insulin assay.

Experiment 6. Effect of intraperitoneal glucose dose on the insulin secretion mechanism

Eight male rats weighing 300 gm were divided into two groups. One group was intraperitoneally injected with 250 mg glucose per 100 gm body weight, the other group was injected with saline solution with the same molarity as the glucose solution. After 30 minutes, the rats were decapitated and the pancreas was excised from each rat. The islets were isolated from each pancreas, and pooled in 2 ml Krebs-Ringer bicarbonate buffer, then evenly divided into two parts. Following the same procedure as Experiment 5, they were glucose or non-glucose

incubated. After centrifuging, the islets were separated from the media and ashed for zinc analysis, and the media after glucose incubation were ready for biological insulin assay.

Chemical Analysis

Zinc content in the islets of Langerhans of the pancreas

The isolated islets were put into an acid washed crucible and ashed in a muffle furnace by slowly raising the temperature to 600° C and allowing to remain at this temperature for 12 hours. The resulted clean ash was dissolved by adding 1 ml of 0.2 N HCl, then zinc content was measured by atomic absorption spectrophotometer.

Zinc content in serum

Serum. The blood drawn from the rat was put into an acid washed test tube. After the blood had coagulated, the clot was removed and the serum spun in centrifuge at 1500 RPM for 15 minutes, then the cell free serum was poured into an acid washed test tube.

Zinc analysis. 200λ of serum was put into an acid washed test tube, into which 400λ of Trichloroacetic acid (TCA) was added. After the mixture had centrifuged for five minutes, the supernatant was aspirated directly for zinc analysis by atomic absorption spectroscopy.

Total and oxidized glutathione

According to Tietze et al. (1969), weighed amounts of tissues were homogenized in five percent TCA/0.01N HCl (1:10 dilution) for one to two minutes at 0° C. After centrifugation (17,000 g for 15 minutes at 2°C), the supernatant solutions were extracted five minutes at 0°C with equal volumes of ether and divided into two portions. (The residual traces of ether were removed by vigorous shaking under

a water pump). One portion was used without further treatment for the determination of total glutathione. The other portion was incubated for one hour at 25°C with an equal volume of 0.04 M NEM (N-ethylmaleimide) in phosphate-EDTA buffer (neutral PH). After removal of unreacted NEM by ten extractions (at least) with ether, the solution was assayed for GSSG.

Components were dissolved in phosphate-EDTA buffer, PH 7.5, and were added in the amounts, and in the order indicated in Table 2.

Table 2. Glutathione assay system

Test Cuvet	Amount	Blank Cuvet
buffer		buffer
DTNB	0.6 μmole	DTNB
GSH or GSSG	1-100 ng	----
Glutathione Reductase	10 μg	Glutathione Reductase
TPNH	0.2 μmole	TPNH

Final volumes were 1.0 ml. The rate of reaction at 25°C was usually expressed as the change in absorbancy per six minutes at 412 mμ (double-beam optics).

By intraploting the absorbancy of the unknown sample in the standard curve, the concentration of the unknown sample was read.

Biological insulin assay

0.5 ml of the incubation media containing insulin was injected into the rat, then blood was withdrawn after 20 minute, 40 minute, and 60 minute periods seperately for glucose assay (Folin et al., 1920). The insulin response was expressed as Glucose (mg/100 ml) per 100 gm body weight by per mg ash weight of islets.

Glucose assay

1. To 0.4 ml acid tungstate solution, add 60 µl of rat blood or each standard. Mix well. If a precipitate forms, centrifuge.

2. Withdraw 200 µl of the clear solution or supernatant and add to 200 l alkaline copper solution in another test tube. Mix well. Cap tightly and boil for eight minutes in a water bath.

3. When cool, add 200 µl of phosphomolybdic acid solution. Mix well, add 2 ml of water, and mix again.

4. Read at 420 m .

Reagents. 1. Acid tungstate solution: Mix one part 10 percent sodium tungstate, one part 2/3 normal sulphuric acid, and seven parts distilled water.

2. Alkaline copper solution: Dissolve 40 g of pure anhydrous sodium carbonate in about 400 ml of water and transfer to a liter flask. Add 7.5 g of tartaric acid, and when the latter has dissolved add 4.5 g of crystallized copper sulfate. Mix and make up to a volume of 1 liter. If the chemicals used are not pure, a sediment of cuprous oxide may form in the course of one or two weeks. If this should happen, filter. To test for the absence of cuprous copper in the solution, transfer 1 ml to a test tube and add 1 ml of the phosphomolybdic acid solution. The deep blue color of the copper should almost vanish.

3. Phosphomolybdic acid solution: To 35 g of molybdic acid and 5 g of sodium tungstate, add 200 ml of ten percent sodium hydroxide and 200 ml of water. Shake vigorously for 20 to 40 minutes to remove the amonia present in the molybdic acid. Cool, dilute to about 350 ml and add 125 ml of concentrated (85 percent) phosphoric acid. Dilute to 500 ml.

Statistical Analysis

Statistical analyses were carried out by analysis of variance. The difference of the mean values were compared by means of F test. Student 't' test was used for pair-comparison between zinc-deficient group and zinc-supplemented pair-fed group, and between glucose and non-glucose incubation groups.

RESULTS AND DISCUSSION

Experiment 1. Effect of Intraperitoneal Glucose Dose on Zinc Content of the Pancreas

Table 3. Effect of IP glucose dose (250 mg/100 gm B.W.) on zinc content in the pancreas 30 minutes after injection

Treatment	No. of Rats	Zinc µg/gm Wet Pancreas	Zinc µg/gm Dry Pancreas
IP-glucose-dosed	6	62.1 ± 29.1	148.7 ± 60.5
Non-glucose-dosed	6	64.1 ± 21.2	149.6 ± 55.2

Values are mean ± SD.

When analyzing pancreatic zinc levels, no differences were found in total pancreatic zinc levels in rats dosed with glucose or non-dosed. Thus I decided to look at the isolated islets.

Experiment 2. Effect of in vitro Glucose Incubation on Zinc Content in the Islets of Langerhans of the Pancreas

In this experiment the insulin secretion from the pancreas or islets was determined after in vitro incubation with glucose for short periods of time. This method has been criticized by some workers on

grounds of interference by lytic substances from the exocrine pancreas. But Gershoff et al. (1973) had shown there was little, if any, comtamination of lytic materials in incubation media during a short incubation period, hence this concern may be excluded.

As summarized in Table 4, there was a significant difference of

Table 4. Zinc content in the islets of Langerhans of the pancreas after incubated with glucose or without glucose

Group	Replicates	Zinc μg/gm Dry Islet[1]	Zinc μg/gm ashed Islet
[2]A	5	29.3 ± 8.2	45.4 ± 19.1
B	5	4.7 ± 2.3	6.4 ± 3.1
C	5	5.0 ± 1.4	8.0 ± 3.4
student's t test:	A vs. B	$p < 0.01$	A vs. B $p < 0.01$
	A vs. C	$p < 0.01$	A vs. C $p < 0.01$
	B vs. C	NS	B vs. C NS

[1]Mean ± SD

[2]A group--pancreas incubated without glucose, followed by isolation of islets

B group--pancreas incubated with glucose, followed by isolation of islets

C group--isolate the islets, then incubate with glucose

zinc content in the islets measured on dry or ashed islet weight between both A and B groups, A and C groups, but not between B and C groups.

Therefore, glucose incubation caused a movement of zinc out of the islets whether they were isolated before or after incubation. These findings agree with the results of Toroptsev et al. (1972) and Engelbart et al. (1970), that the zinc content in beta cells is reduced on glucose stimulation, while the alpha cells only change slightly.

Experiment 3. Effect of Zinc Deficiency on Glutathione Content in the Pancreas and on Zinc Movement from the Islets of Langerhans in Response to Glucose Stimulation

The daily weight gains (mean ± SD) were: 1.4 ± 0.7 g for the zinc-deficient group; 1.7 ± 0.6 g for the zinc-supplemented pair-fed group; 5.7 ± 1.9 g for the zinc-supplemented ad libitum control. The difference of daily weight gain between zinc-deficient and zinc-supplemented pair-fed groups was not significantly different, but it was significantly different ($p < 0.01$) between these two groups and zinc-supplemented ad libitum control. The daily food intakes (mean ± SD) were: 8.3 ± 1.2 g for zinc-deficient and zinc-supplemented pair-fed groups; 16.5 ± 0.8 g for zinc-supplemented ad libitum control. The zinc-deficient group showed a cyclic pattern of food intake. After four weeks, the zinc level in serum (mean ± SD) were: 65.0 ± 14.1 μg/100 ml for zinc-deficient group, 151.3 ± 54.2 g/100 ml for zinc-supplemented pair-fed group; 303.0 ± 28.9 μg/100 ml for zinc-supplemented ad libitum control.

In order to eliminate the inanition factor which accompanies zinc-deficiency, paired feeding of the zinc-supplemented group was used. The cyclic pattern of food intake in zinc-deficient rats was disregarded, since Gershoff et al. (1973) reported no statistical correlation had been observed between the cyclic food intake of zinc-deficient rats and their serum glucose levels.

The zinc-deficient rats lost their appetite very fast, with a poor daily food consumption only half as much as that consumed by zinc-supplemented ad libitum rats; also they developed a cyclic pattern of food intake, which had a frequency of 3.6 days. This syndrome has been observed by other workers (Hendricks et al., 1972 and Gershoff et al., 1973).

As shown in Appendix C and D, the growth of zinc-supplemented ad libitum fed rats was steady and continuous during the entire experimental period, while zinc-deficient and zinc-supplemented pair-fed rats grew rapidly only during the first week, with a decreasing growth rate during the subsequent periods. Although there was more growth in zinc-supplemented pair-fed rats than zinc-deficient ones, no significant differences were found at each week period.

Serum zinc level was markedly reduced in zinc-deficient rats. It was half of that of zinc-supplemented pair-fed group, and 20 percent of that of zinc-supplemented ad libitum group.

As summarized in Table 5, there was significant differences of zinc contents in the islets ($p < 0.01$) between zinc-deficient and both zinc-supplemented groups after glucose incubation. No significant difference was found in the three groups incubated without glucose,

Table 5. Zinc content in the islets of Langerhans of the pancreas after incubation with glucose or without glucose in the rats fed with different diets

Group	No. of Rats	Treatment	Zinc[1] μg/gm Ashed Islet
Zinc-deficient	14	[2]-A	87.1 ± 31.8
		-B	54.3 ± 17.4
Zinc-supplemented pair-fed	13	+A	27.6 ± 8.9
		+B	65.6 ± 29.8
Zinc-supplemented ad libitum	10	A	31.8 ± 19.1
		B	83.6 ± 35.0
student's t test:		-A vs. +A	$p < 0.01$
		-B vs. +B	NS
		A vs. B	$p < 0.01$
		-A vs. -B	NS
		+A vs. +B	$p < 0.01$
F test:		-A vs. A	$p < 0.01$
		+A vs. A	NS
		-B vs. B	NS
		+B vs. B	NS

[1]Mean ± SD

[2]-A--zinc-deficient, glucose incubation
-B--zinc-deficient, non-glucose incubation
+A--zinc-supplemented pair-fed, glucose incubation
+B--zinc-supplemented pair-fed, non-glucose incubation
A--zinc-supplemented ad libitum, glucose incubation
B--zinc-supplemented ad libitum, non-glucose incubation

although the levels of zinc contents in the islets of both zinc-deficient and zinc-supplemented pair-fed rats were lower compared with zinc-supplemented ad libitum control. It therefore clearly showed up on glucose incubation that zinc moved out of the islets in both zinc-supplemented rats, but not in zinc-deficient rats. In other words, there was an impairment of zinc movement from the islets on glucose stimulation in zinc-deficiency. It is interesting to correlate this finding with the results obtained by Gershoff et al. (1973) that insulin released from zinc-deficient pancreatic slices during in vitro glucose incubation was lower than those of zinc-supplemented pair-fed and zinc-supplemented ad libitum controls.

Because of high standard errors which occurred in calculations of zinc values in Experiment 3, it was advisable and challenging to further investigate the relationship between zinc deficiency and insulin release, thus Experiment 5 was conducted.

Gershoff et al. (1973) suggested that there were two possibilities for the reduced level of circulating insulin in zinc-deficient rats: (1) decreased insulin release from the pancreas or; (2) increased breakdown or uptake of circulating insulin. Hsu et al. (1968) found that the concentrations of non-protein SH compounds and glutathione in the livers of zinc-deficient rats were significantly ($p < 0.01$) higher than those of zinc-supplemented controls. However, Hendricks et al. in their recent unpublished work, found during in vitro incubation of insulin with liver homogenate of rats, there were no significant differences of insulin degradation between zinc-deficient and zinc-supplemented rats. Therefore it is unlikely that glutathione in the liver of zinc-deficient rats will promote increased insulin degradation.

To the concern that there may be an increased amount of glutathione in the pancreas in zinc-deficiency, which is responsible for the decreased insulin release from the islets, glutathione contents in the pancreas of zinc-deficient rats were measured in Experiment 3. Surprisingly, there were no significant differences between zinc-deficient and both zinc-supplemented groups in the amounts of either total or oxidized glutathione (Table 6). Again it indicates that the reduced insulin secretion in zinc-deficient rats was not due to the promotion of insulin degradation by the glutathione in the pancreas.

Table 6. Oxidized and total glutathione content in the pancreas of the rats fed with different diets

Group	No. of Rats	Glutathione Content[1] (μg/gm Dry Pancreas)	
		Oxidized Form	Total
Zinc-deficient	14	34.6 ± 4.4	593.5 ± 61.3
Zinc-supplemented pair-fed	13	38.4 ± 29.6	635.9 ± 60.2
Zinc-supplemented ad libitum	10	34.1 ± 27.7	579.2 ± 68.3

[1]Mean ± SD

Experiment 4. Effect of in vitro Glucose Incubation on Insulin Release from the Islets of Langerhans

A measurement of insulin activity can be obtained by injecting the supernatant incubation media from the isolated islets into a rat and measuring the blood glucose depression.

The curves in Figure 1 showed, when injecting with the glucose-incubated media there was a rapid drop to a minimum -4.88 mg/100 ml

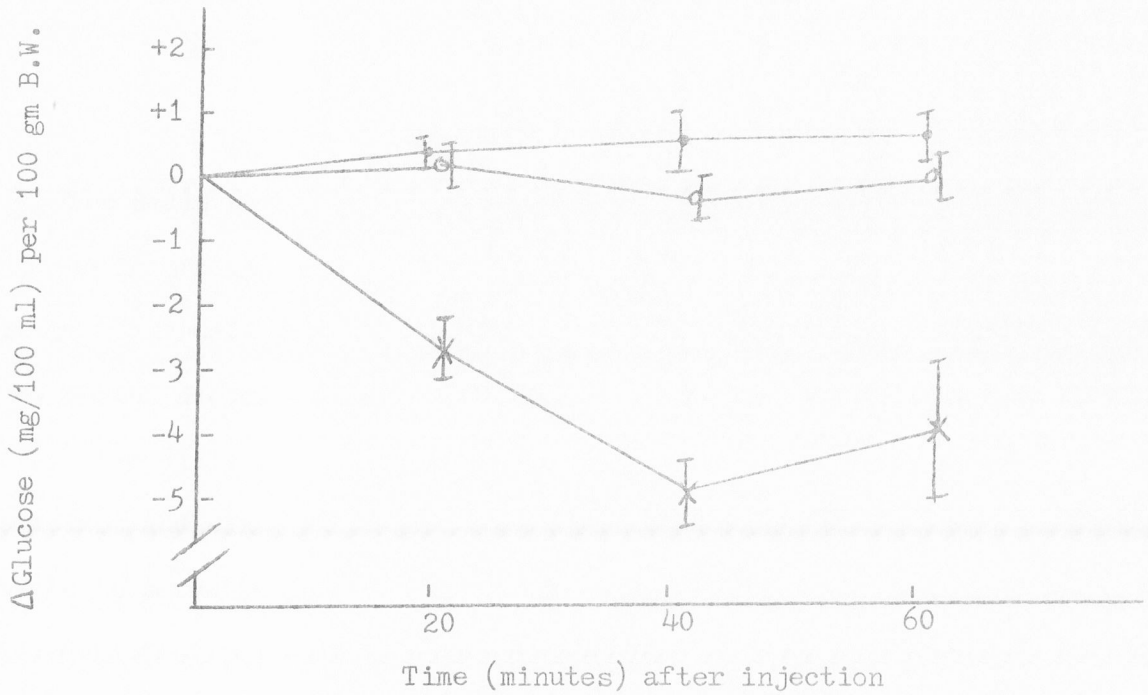

Figure 1. Changes of the blood glucose level of the rats after injections of the various media; media with glucose incubation (x——x), media without glucose incubation (o——o), buffer containing glucose (•——•). Vertical bars are standard errors.

per 100 gm B.W. 40 minutes after injection, then a small rise 60 minutes after injection, while the changes of glucose concentrations after injection of the other two media were very small. The glucose values on each interval were significantly ($p < 0.01$) different between glucose incubation group and the other two groups, but the differences between non-glucose incubation and buffer containing glucose groups

were not significant (see Appendix G). It indicates only the supernatant from islets incubated with glucose solution contained insulin which had been released from the isolated islets.

Experiment 5. Effect of Zinc Deficiency on the Insulin Secretion Mechanism

The daily weight gains (mean ± SD) were: 2.1 ± 0.9 g for the zinc-deficient group; 2.3 ± 0.7 g for the zinc-supplemented pair-fed group. Zinc-deficient rats decreased the food intake within three days. They showed a cyclic variation of food intake with a frequency of 3.2 days. The daily food intakes (mean ± SD) were: 7.3 ± 1.4 g for zinc-deficient and zinc-supplemented rats. After three weeks, the zinc levels in serum (mean ± SD) were: 52.4 ± 9.2 μg/100 ml for zinc-zinc-deficient rats; 103.0 ± 47.5 μg/100 ml for zinc-supplemented pair-fed rats.

As seen in Appendix E and F, the zinc-deficient and zinc-supplemented pair-fed rats grew rapidly in the first week, but began to greatly reduce the growth rate at the second week.

It was observed in Table 7 that the zinc content of islets incubated without glucose in the zinc-deficient group was significantly ($p < 0.01$) lower than that of the zinc-supplemented pair-fed group. This is different from the results obtained in Experiment 3. With a much less standard error, these data may be more acceptable. The difference between glucose incubation and non-glucose incubation group were significantly different in the zinc-supplemented pair-fed group, but not in the zinc-deficient group. This is consistent with what

Table 7. Zinc content in the islets of Langerhans in zinc-deficient and zinc-supplemented pair-fed rats after incubated with or without glucose

Group	No. of Rats	Treatment	Zinc μg/gm Ashed Islet[1]
Zinc-deficient	12	[2]-A	33.83 ± 4.10
		-B	39.96 ± 6.14
Zinc-supplemented pair-fed	12	+A	56.52 ± 5.21
		+B	69.72 ± 7.35
student t test:		-A vs. -B	NS
		+A vs. +B	$p < 0.01$
		-A vs. +A	$p < 0.01$
		-B vs. +B	$p < 0.01$

[1]Mean ± SD
[2]-A--zinc-deficient, glucose incubation
-B--zinc-deficient, non-glucose incubation
+A--zinc-supplemented pair-fed, glucose incubation
+B--zinc-supplemented pair-fed, non-glucose incubation

was found in Experiment 3. It showed again there was in aimpairment of zinc movement from the islets on glucose stimulation in zinc-deficient rats.

To clarify the changes of blood glucose level after injections of the incubation media, two ways were expressed; one was based on per 100 gm body weight, the other was based on per 100 gm body weight by per mg of ashed islet. Both curves showed the same shape, so that only the changes of blood glucose level based on per 100 gm body weight by per mg ashed islet weight were presented in Figure 2.

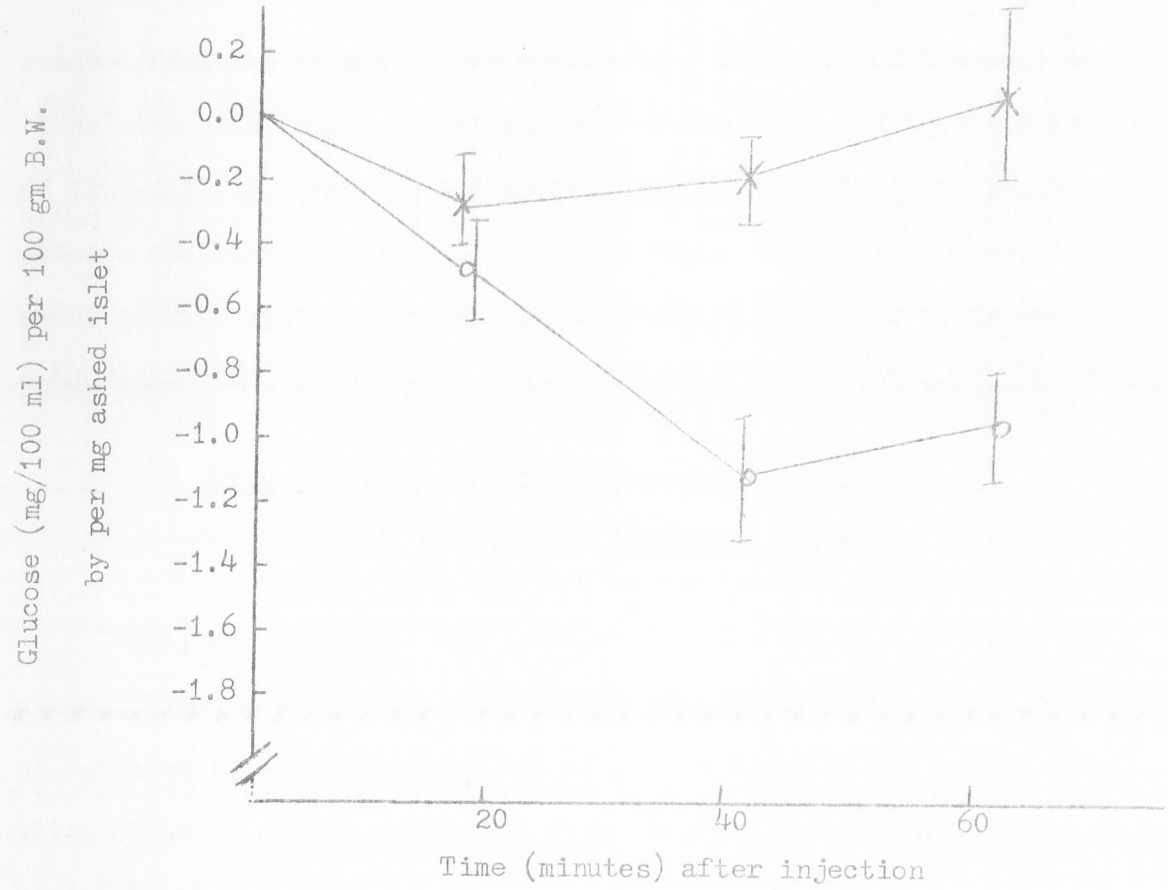

Figure 2. Changes of the blood glucose concentration after injections of glucose-incubated media; zinc-deficient group (x____ x), zinc-supplemented pair-fed group (o____ o). Vertical bars are standard errors.

The curve for the media glucose-incubated with islets from the zinc-supplemented pair-fed rats showed a more rapid drop after 20 minutes than that of the zinc-deficient group. The values of the zinc-supplemented pair-fed group at the 40 minute and 60 minute intervals were significantly ($p < 0.01$) lower than those of the zinc-deficient group, but not at the 20 minutes interval (see Appendix H). It indicates that more insulin was released from the islets of zinc-supplemented rats.

From the results above, it is possible to postulate that zinc is removed from the islets of Langerhans as a component of insulin, or along with insulin release on glucose stimulation, and that the islets of zinc-deficient rats do not secrete as much insulin in response to a glucose challenge as zinc-supplemented rats. This raised questions about in vivo systems and insulin release, so a further study was conducted.

Experiment 6. Effect of Intraperitoneal Glucose Dose on the Insulin Secretion Mechanism

Thirty minutes after the intraperitoneal glucose injection, the average blood glucose level of the rats was elevated to 241.8 mg/100 ml compared to a much lower level of 71.0/100 ml for the saline dosed rats. This is rather consistent with the results obtained by Hendricks et al. (1972).

As observed in Table 8, the zinc contents in the islets incubated with glucose were less than those in the islets incubated without glucose in both IP-glucose-dosed and saline-dosed groups, and there showed a greater reduction in saline-dosed group compared with IP glucose dosing of rats 30 minutes prior to sacrificing followed with the isolation of islets caused a marked depression in the zinc movement from isolated islets when incubated with glucose in vitro. However, in vivo dosing did not depress zinc content in islets significantly (B vs. D).

Figure 3 illustrated that there was more insulin present in the incubation media of saline-dosed group than in the IP-glucose-dosed

Table 8. Zinc content in the islets of Langerhans of the pancreas in IP-glucose-dosed and saline-dosed rats after incubated with or without glucose

Treatment		No. of Rats	Zinc, μg/gm Ashed Islets[1]
IP-glucose-dosed	A[2]	4	61.72 ± 6.33
	B		76.31 ± 5.51
Saline-dosed	C	4	45.24 ± 4.12
	D		85.47 ± 6.02

student's t test:	A vs. B	$p < 0.05$
	C vs. D	$p < 0.01$
	A vs. C	$p < 0.01$
	B vs. D	NS

[1] Mean ± SD
[2] A--IP-glucose-dosed, glucose incubation
B--IP-glucose-dosed, non-glucose incubation
C--saline dosed, glucose incubation
D--saline dosed, non-glucose incubation

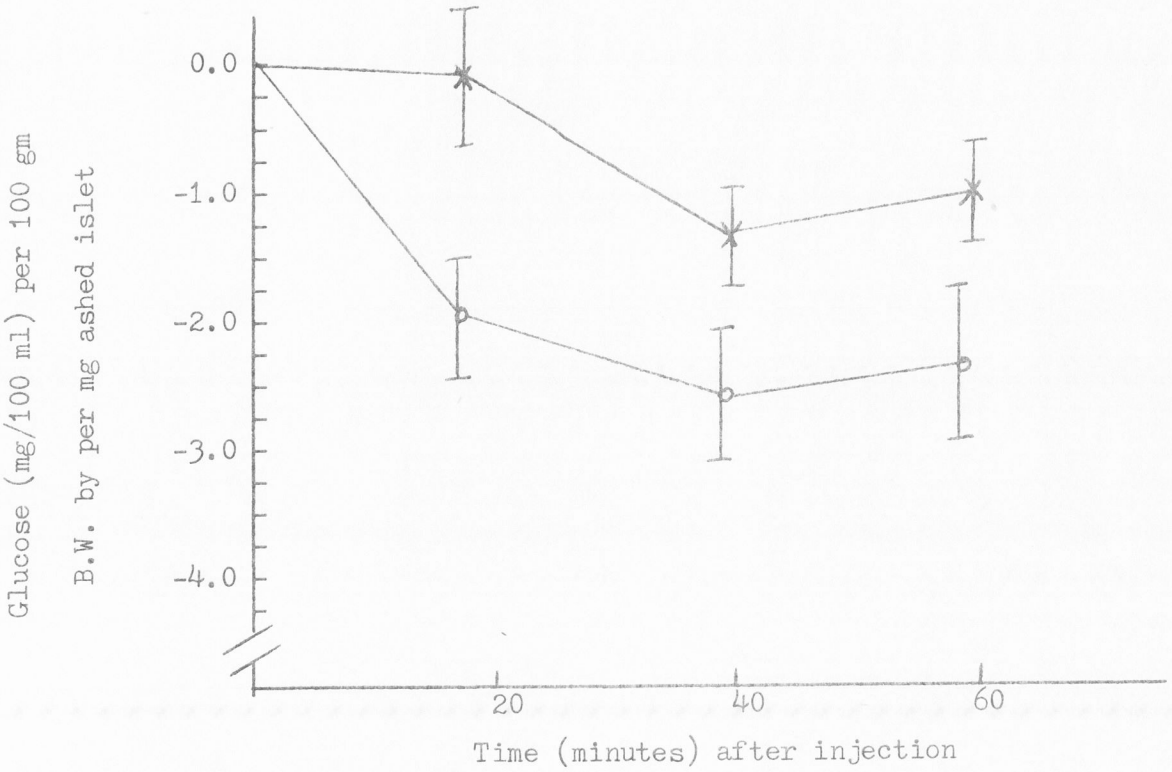

Figure 3. Changes of the blood glucose concentration after injection of the glucose-incubated media. IP-glucose-dosed (x——— x), saline-dosed (o——— o). Vertical bars are standard errors.

group. The values of IP-glucose-dosed and saline-dosed groups were significantly different ($p<0.05$) at each interval (see Appendix I). It indicates that insulin secretion was significantly reduced when the isolated islets were taken from the animals previously stimulated by glucose in vivo.

The results from the previous experiments indicated that zinc follows insulin in the secretory process, however, whether as a component of insulin or along with insulin secretion in unknown. In zinc-deficiency status, accompanied with a decreased serum zinc level in the rat, there is a reduced zinc content in the islets of Langerhans, which may induce the impairment of insulin synthesis or secretion.

Gershoff et al. (1973) suggested zinc-deficiency results in the impaired insulin release, since no reduced pancreatic insulin was found in zinc-deficient rats but only a reduced circulating insulin. However, in this thesis it is suggested that zinc-deficiency may cause the impairment of both insulin synthesis and release. It was reported zinc-deficiency results in a decreased nucleic acid synthesis (Oberleas et al., 1969 and Turk et al., 1966) and Dreosti et al. (1975) reported there is a depressed thymidine kinase activity in zinc-deficient rats. These findings may indicate the impaired insulin synthesis in the islets of Langerhans in zinc-deficiency. On the other hand, it is suggested that the insulin release mechanism may be impaired in zinc-deficiency, due to the activity losses of zinc-dependent enzymes which are necessary for the fussion between the membrane of secretory granule and the beta cell membrane (Satir, 1975).

In Experiment 6 the insulin release on in vitro glucose stimulation was significantly reduced when the animals were glucose-challenged in vivo in advance. This indicates probably there is an inhibition of insulin release after an in vivo glucose challenge.

It is interesting to notice the results obtained in Experiment 1, that there was no zinc movement from the whole pancreas 30 minutes after the intraperitoneal glucose dose. This leaves a question as to what is the role of zinc after it moves out of the islets. More investigations seem to be needed.

SUMMARY AND CONCLUSION

1. Accompanied with an inanition, there is a cyclic pattern of daily food intake with a frequency of 3.6 days in zinc-deficient rats.

2. There is no significant movement of zinc from the pancreas thirty minutes after the intraperitoneal glucose administration.

3. With an in vitro glucose stimulation, there is a reduction of zinc content in the islets of Langerhans of the pancreas. It is concluded that zinc moves from the islets as a component of insulin on glucose stimulation.

4. In zinc deficiency there is a reduced zinc content in the islets of Langerhans of the pancreas.

5. There is a reduced zinc movement and a reduced insulin release from the islets of Langerhans of zinc-deficient rats on in vitro glucose stimulation, an impairment of the mechanism of insulin release or insulin synthesis in zinc deficiency is suggested.

6. Insulin secretion is significantly reduced in the isolated islets taken from animals prestimulated with glucose in vivo.

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APPENDIXES

Appendix A

Table 9. Composition of the mineral mixture in the basal diet

Ingredients	gm/kg of Mineral Mixture
Glucose	234.5
Na_2HPO	323.0
$CaCO_3$	312.0
KCl	86.0
$MgCO_3$	34.7
$FeSO_4 \cdot 7H_2O$	4.3
$MnSO_4$	3.8
$CoCl_2$	0.5
$CuSO_4 \cdot H_2O$	0.4
Na_2MoO_4	0.06
KI	0.05

Appendix B

Table 10. Composition of the vitamin mixture in the basal diet[1]

Ingredients	gm/kg of Vitamin Mixture
Vitamin A concentrate (200,000 IU per gram)	4.5
Vitamin D concentrate (400,000 IU per gram)	0.25
α -Tocopherol	5.0
Ascorbic acid	45.0
Inositol	5.0
Choline chloride	75.0
Menadione	2.25
p-Amino benzoic acid	5.0
Niacin	4.5
Riboflavin	1.0
Pyridoxine hydrochloride	1.0
Thiamine hydrochloride	1.0
Calcium pantothenate	3.0
Biotin	0.02
Folic acid	0.09
Vitamin B	0.0014

[1]Vitamin diet fortification mixture. Nutrition Biochemical Corporation, Cleveland, Ohio.

Appendix C

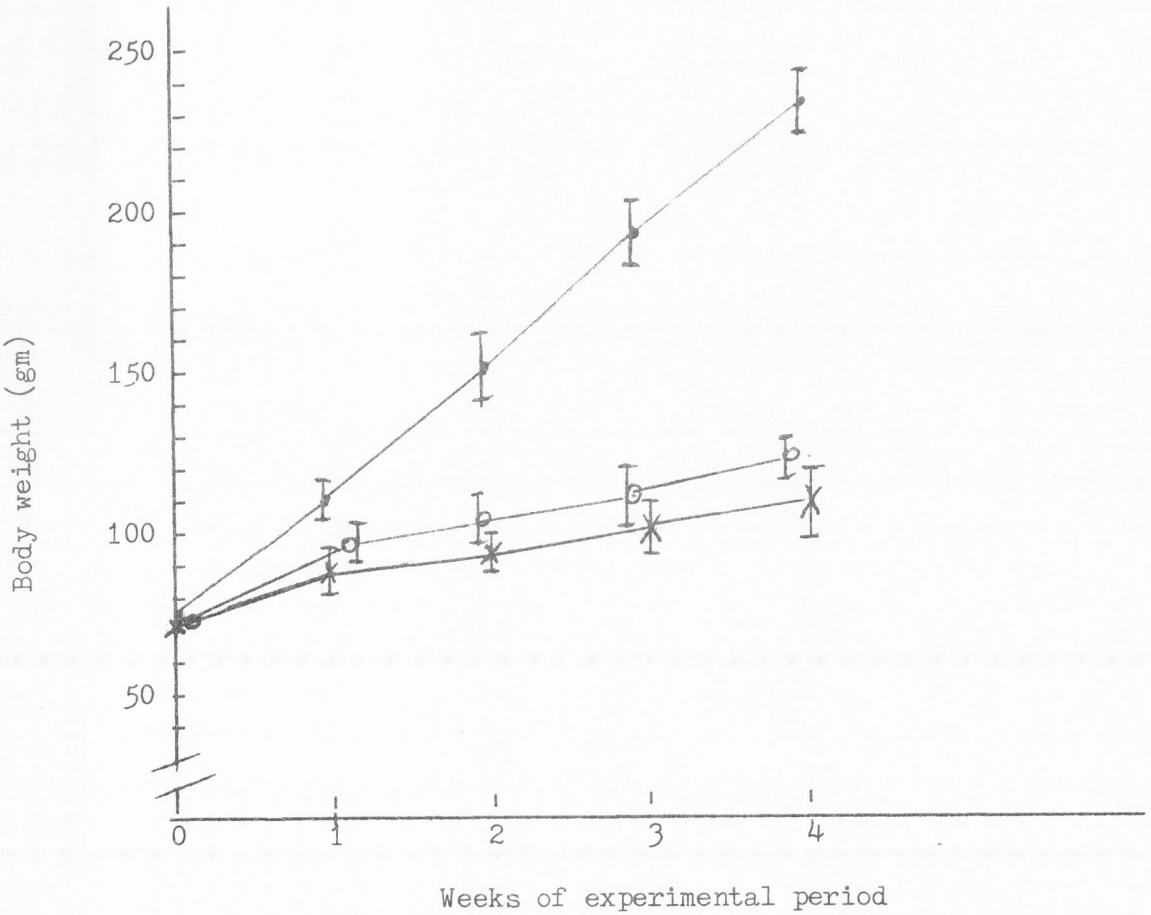

Figure 4. Growth curves of rats fed with zinc-deficient or zinc-supplemented diets in Experiment 3. Zinc-deficient rats (x——x), zinc-supplemented pair-fed rats (o——o), zinc-supplemented ad libitum rats (·—— ·). Vertical bars are standard errors.

Appendix D

Table 11. Body weights of the rats during the experimental period in Experiment 3.

Group	No. of Rats	Body Weight[1] (gm)				
		Week 0	Week 1	Week 2	Week 3	Week 4
Zinc-deficient	14	70.3 ± 5.1^{a}	89.4 ± 7.2^{a}	94.7 ± 3.8^{aa}	101.8 ± 6.3^{aa}	109.0 ± 10.3^{aa}
Zinc-supplemented pair-fed	14	70.6 ± 2.3^{a}	93.7 ± 5.5^{a}	102.7 ± 4.2^{aa}	110.2 ± 8.4^{aa}	121.8 ± 6.9^{aa}
Zinc-supplemented ad libitum	10	73.4 ± 4.5^{a}	111.3 ± 5.8^{b}	150.6 ± 9.0^{bb}	191.2 ± 9.5^{bb}	233.1 ± 9.7^{bb}

[1] Mean $\pm$ SE

Statistics by student t test; means having different superscripts differ significantly, single letter ($p < 0.05$), double letters ($p < 0.01$).

Appendix E

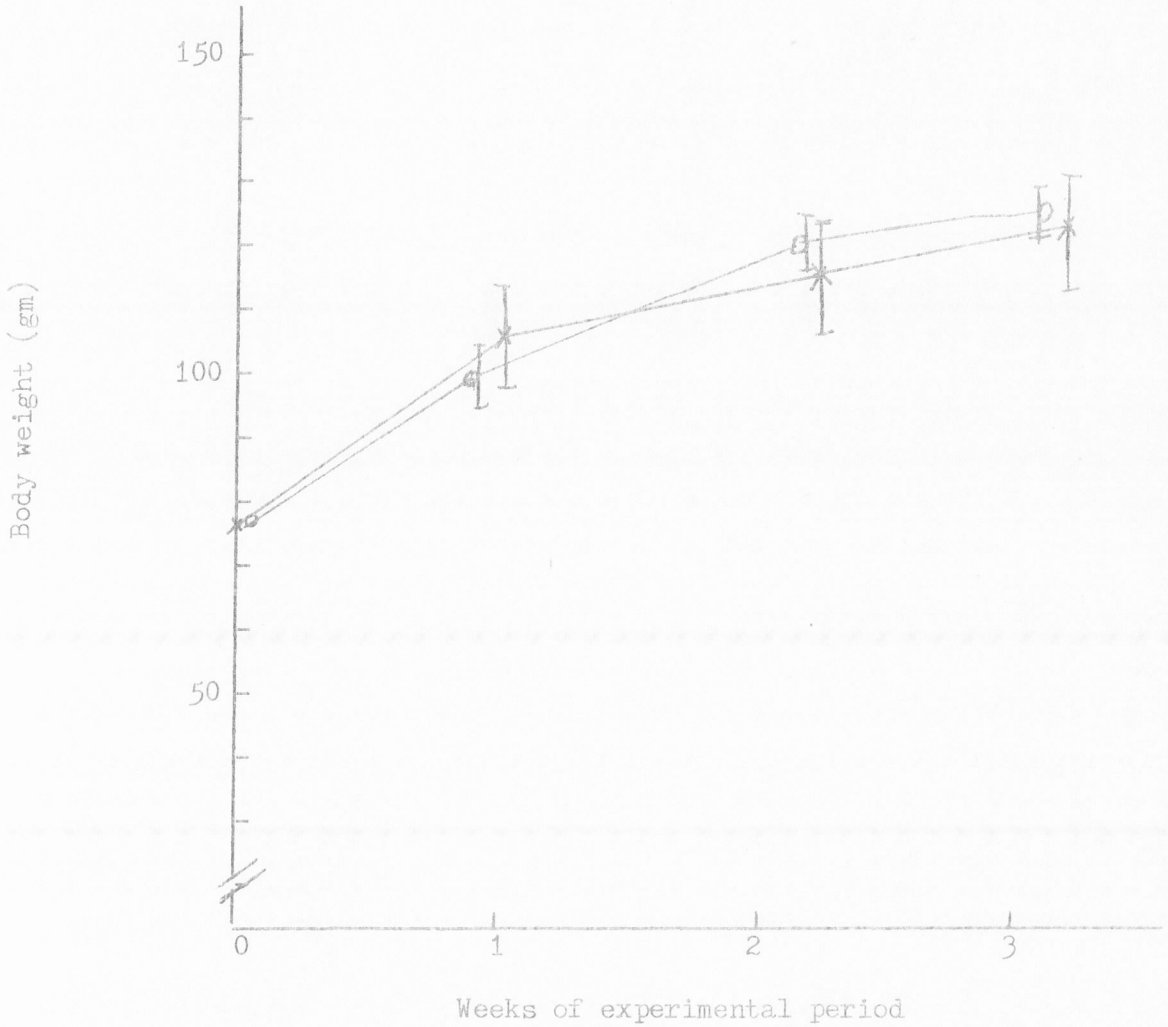

Figure 5. Growth curves of rats fed with zinc-deficient or zinc-supplemented diets in Experiment 5. Zinc-deficient rats (x——x), zinc-supplemented pair-fed rats (o——— o). Vertical bars are standard errors.

Appendix F

Table 12. Body weights of the rats during the experimental period in Experiment 5.

Group	No. of Rats	Body Weight[1] (gm)			
		Week 0	Week 1	Week 2	Week 3
Zinc-deficient	12	77.6 ± 5.8	106.2 ± 9.6	117.0 ± 8.5	127.7 ± 8.1
Zinc-supplemented pair-fed	12	77.8 ± 7.5	100.3 ± 4.3	121.3 ± 3.3	128.0 ± 6.4

[1]Mean ± SE

Appendix G

Table 13. Changes of blood glucose levels after injections of incubation media in Experiment 4

Media Treatment	No. of Rats	Δ Glucose mg/100 ml blood/100 gm B.W.[1]		
		Time after Injection		
		20 minutes	40 minutes	60 minutes
Glucose incubation	3	$-(2.74 \pm 0.45)^{aa}$	$-(4.88 \pm 0.41)^{aa}$	$-(3.89 \pm 1.0)^{aa}$
Non-glucose incubation	3	$+(0.31 \pm 0.25)^{bb}$	$-(0.23 \pm 0.27)^{bb}$	$+(0.20 \pm 0.31)^{bb}$
Buffer with glucose	3	$+(0.34 \pm 0.25)^{bb}$	$+(0.48 \pm 0.50)^{bb}$	$+(0.66 \pm 0.37)^{bb}$

[1] Mean ± SE

Statistics by student t test; means having different superscripts differ significantly ($p < 0.01$) in each column.

Appendix H

Table 14. Changes of blood glucose levels after injections of media glucose incubated with the islets from the pancreas of zinc-deficient and zinc-supplemented pair-fed rats in Experiment 5.

Group	No. of Rats	Δ Glucose mg/100 ml blood/100 gm B.W.[1]		
		Time after Injection		
		20 minutes	40 minutes	60 minutes
Zinc-deficient	12	$-(0.47 \pm 0.11)^{aa}$	$-(0.44 \pm 0.06)^{aa}$	$+(0.21 \pm 0.03)^{aa}$
Zinc-supplemented pair-fed	12	$-(1.28 \pm 0.18)^{aa}$	$-(2.88 \pm 0.33)^{bb}$	$-(2.28 \pm 0.24)^{bb}$
		Δ Glucose mg/100 ml blood/100 gm B.W./mg ashed islet[1]		
Zinc-deficient	12	$-(0.30 \pm 0.06)^{aa}$	$-(0.20 \pm 0.02)^{aa}$	$+(0.08 \pm 0.02)^{aa}$
Zinc-supplemented pair-fed	12	$-(0.46 \pm 0.06)^{aa}$	$-(1.18 \pm 0.14)^{bb}$	$-(0.94 \pm 0.10)^{bb}$

[1] Mean ± SE

Statistics by student t test; means having different superscripts differ significantly ($p < 0.01$) in each column.

Appendix I

Table 15. Changes of blood glucose levels after injection of media glucose-incubated with the islets from the pancreas of IP-glucose-dosed and saline-dosed rats in Experiment 6.

Group	No. of Rats	Δ Glucose mg/100 ml blood/100 gm B.W.[1]		
		Time after Injection		
		20 minutes	40 minutes	60 minutes
IP-glucose-dosed	4	$-(1.85 \pm 0.29)^{aa}$	$-(3.61 \pm 0.25)^{aa}$	$-(2.94 \pm 0.27)^{aa}$
Saline-dosed	4	$-(4.18 \pm 0.18)^{bb}$	$-(5.84 \pm 0.36)^{bb}$	$-(5.16 \pm 0.43)^{bb}$
		Δ Glucose mg/100 ml blood/100 gm B.W./mg Ashed Islet[1]		
IP-glucose-dosed	4	$-(0.74 \pm 0.14)^{a}$	$-(1.44 \pm 0.18)^{a}$	$-(1.18 \pm 0.16)^{a}$
Saline-dosed	4	$-(1.92 \pm 0.42)^{b}$	$-(2.68 \pm 0.56)^{b}$	$-(2.42 \pm 0.56)^{b}$

[1] Mean ± SE

Statistics by student t test; means having different superscripts differ significantly single letter ($p < 0.05$), double letters ($p < 0.01$).

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